Amino Acid Profiling by Reactive Desorption Electrospray Ionization Tandem Mass Spectrometry (DESI-MS/MS)

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ABSTRACT

Desorption electrospray ionization mass spectrometry (DESI MS) has gained significant recognition over the past few years because of its simplicity and rapid throughput capabilities, allowing for the direct analysis of samples with a wide variety of sizes, shapes, and chemistries. Addition of various reagents into the DESI spray solvent enables atmospheric pressure ion molecule reactions between these reagents in the charged micro droplets and analyte molecules on the sample surface affording improved selectivity and sensitivity in some cases. Presented is a rapid screening method for amino acids (aas) based on reactive DESI. Amino acids have been shown to play key roles in the regulation of cellular processes. They are also particularly vital in the determination of metabolic disorders such as phenylketonurea, homocystinuria, and tyrosinemia. The most specific and reliable methods for diagnosing these disorders are based on the determination of aas in body fluids using methodologies such as gas/liquid chromatography, tandem MS, and various combinations thereof. However, these methods are usually time-consuming, increasing the time physicians wait before administering treatment or regulating the diet of diseased infants.

The reactive DESI approach presented here is based on the formation of stable noncovalent complexes between alpha-cyclodextrin (dissolved in the spray solvent) and amino acids present in the sample affording a selective method for their detection. However, the selectivity and sensitivity of screening for aas was improved by performing the MS analysis in the multiple reaction monitoring mode when using a quadrupole ion trap or by the precursor ion scan when using a triple quadrupole MS instrument, affording an average twenty-five times sensitivity improvement compared to analysis in

full scan mode. The observation of similar complexes with various carboxylic acids including formic acid and acetic acid, and evidence from tandem MS experiments indicate that amino acid:alpha-cyclodextrin complexation reactions occur by hydrogen bonding interaction with carboxyl group of the aas. The specificity and sensitivity provided by this approach seems very promising for applications in the rapid screening of aas directly from body fluids including urine and plasma for amino acid disorders in a clinical setting.

KEYWORDS

Ambient Mass Spectrometry, Desorption Electrospray Ionization, Amino Acid Disorders, Multiple Reaction Monitoring

INTRODUCTION

Desorption electrospray ionization mass spectrometry (DESI MS) is a relatively new analytical method that allows rapid analysis times with little sample preparation. In DESI MS, a pneumatically assisted electrospray plume is continuously sprayed towards the sampling surface, forming a thin, micron-size solvent film on the sample. Formation of this solvent film allows rapid extraction and/or dissolution of analyte molecules from the sample surface. The continuous flow of the electrospray plume momentarily replaces the existing solvent film by generating analyte-containing secondary droplets. The secondary droplets are then directed towards the inlet of the mass spectrometer.

Although quantitative chemical analysis is not easily achievable by analysis of the untreated sample, the addition of an internal standard enables quantifiable results to a significantly accurate degree.[1] Specifically, DESI's rapid analysis of compounds, both biological and inorganic, can be used in the fast turnaround time analysis for the diagnosis of blood-related disorders in clinical settings such as the screening for aa related disorders.[2] Amino acids are the building blocks of proteins and have many functions in the body. Hereditary disorders of amino acid processing can be the result of defects either in the breakdown of amino acids or in the body's ability to get the amino acids into cells.[4, 5] The buildup of amino acids and/or by-products of amino acid metabolism in the blood can cause severe medical complications.[6] Since these disorders produce symptoms early in life, newborns in the United States and abroad are routinely screened for several common disorders, such as phenylketonuria, maple syrup urine disease, homocystinuria, tyrosinemia, and a number of other inherited disorders.[7] Urine and blood tests used to diagnose the amino acid disorders utilize thin layer

chromatography to separate the amino acids present. Using this technique, amino acids form characteristic patterns on a glass plate coated with a thin layer of silica gel. The experimental pattern is then compared to a standard pattern to determine if there are abnormalities.[8, 9]

The presentation of the various aminoacidopathies varies from no obvious clinical symptoms for months (phenylketonuria), to acute encephalopathy (maple syrup urine disease) within days following birth.[10] In each of these disorders, the lack of early identification and treatment may result in serious medical consequences including mental retardation, developmental delays, failure to thrive, and death.[11] Treatment of these disorders is accomplished with dietary restriction of the specific amino acids and medication.[12] Urea cycle disorders require treatment with low protein diets and medications to prevent hyperammonemia and remove toxic compounds. Infants with neonatal presentations of a urea cycle disorders represent medical emergencies with varying outcomes.[13] Patients typically require aggressive treatment with hemodialysis, which can be both painful and costly.[14]

The present investigation reports the application of DESI MS in the reactive mode for the rapid detection of amino acids. Analysis of amino acid standards by reagent free DESI MS, in positive and negative ion mode resulted in an overall higher sensitivity in negative mode than positive mode, with the exception of the basic aas which showed higher sensitivity in positive ion mode. In an effort to improve the selectivity for the detection of aas, similar experiments were explored in reactive DESI mode by the addition of α-cyclodextrin into the spray solvent resulting in the formation of stable gas phase complexes. Insights into the gas phase structure of the complexes was evaluated by

tandem MS analysis and by performing electrospray experiments of cyclodextrin solutions containing various carboxylic acids including formic and acetic acid. The selectivity and sensitivity for the simultaneous detection of various amino acids in the reactive DESI experiments was further enhanced by either performing MS analysis in the multiple reaction monitoring mode on a quadrupole ion trap mass spectrometer or the precursor ion scan using a triple quadrupole instrument, affording a 25x improvement in sensitivity when compared to analysis in the full scan mode.

EXPERIMENTAL

Samples and reagents

All reagents were used without additional purification. HPLC grade methanol (EMD Chemicals, Gibbstown, NJ, USA) and acetonitrile (EMD Chemicals, Gibbstown, NJ, USA), were used as spray solvents in the DESI experiments. HPLC grade methanol and ultrapure water (18.2 MΩ cm^{-1}), obtained from a Nanopore purification unit (Barnstead, San Jose, CA, USA), were used in the amino acid standard preparation of the 50:50 MeOH:H_2O solutions. Individual crystallized amino acid samples (Sigma-Aldrich, St. Louis, MO, USA) were used to make the stock solutions. The stock solutions were made to a final concentration of 10 mM in a 50:50 MeOH:H_2O mixture. The reagentless DESI experiments used 100% MeOH as the spray solvent. Unless otherwise stated, 30 μM α-cyclodextrin (TCI America, Portland, OR, USA) in 50:50 ACN:MeOH was used as the spray solvent for the reactive DESI experiments. Industrial grade N_2 (99.998% Airgas, Atlanta, GA, USA) was used for the nebulizer gas sprayer.

DESI ion source

A home-built DESI ion source (Figure 1) consisting of a manual X-Y and Z sample stage (Thorlabs, Newton, NJ) and a high-performance sprayer [16] was used for all experiments. The sprayer was fixed to a post which was mounted on a MicroBlock 3-axis positioner (Thorlabs, Newton, NJ) allowing manual adjustments of the emitter position in the XYZ coordinates, respective to the capillary inlet. This sprayer holder assembly was secured to a bread board (Thorlabs, Newton, NJ) to ensure minimal undesired movement.

The X-Y stage which allows sample translation in the x-y axis was mounted atop a heavy duty lab jack (Thorlabs, Newton NJ) for manual adjustment of the sample position in the *z*-axis. The lab jack-stage assembly was also affixed to the bread board. Samples were held in place using two Al sample holders (110 x 10 x 3 mm) mounted on the sample plate of the microscope stage. The sprayer tip was positioned 2–3 mm from the capillary inlet and 0.1-1 mm away from the surface of the sample at an angle of 55^{o}. The collection angle was set ~10^{o}. The DESI spray solutions were delivered to the sprayer at a flow rate of 5 µL/min via a syringe pump and a 250 mL glass syringe (Hamilton Company, Reno, NV). Solvents were administered through two pieces of PTFE tubing (127 mm id) connected through a T union with one end connected to the sprayer and the other end to the syringe. Spray solvents were charged to +/-3 kV (depending on positive or negative ion mode) by an external high voltage power supply (Stanford Research Systems Inc., Sunnyvale, CA). The power supply was in electrical contact with the spray solution through one port of a T union. The N_2 nebulizer gas pressure was set to 110 psi.

Mass Spectrometry

DESI MS experiments were performed on a Thermo Finnigan LCQ Deca *XP*+ quadrupole ion trap[2] (Thermo Fisher Scientific, San Jose, CA, USA) and on an Agilent 6410 series triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The standard ion transfer capillary of the ion trap instrument was replaced with an extended version (20.5 cm long, 762 µm i.d., 1588 µm o.d. Small Parts Inc., Miramar, FL, USA) and a similar stainless steel capillary was coupled to the standard ion transfer

capillary of the QqQ instrument through a custom made Vapur-type interface to enable DESI MS investigations. Initial optimization experiments for the evaluating of the individual aas and mixtures of those commonly presented in aa disorders were performed by reagentless and reactive DESI on the QiT MS instrument. Selectivity and sensitivity improvements for the targeted analysis of amino acid mixtures, was achieved following preprogrammed MRM transitions on the QiT instrument and compared with similar analysis on a triple quadrupole instrument using a precursor ion scan experiments.

RESULTS and DISCUSSION

Analysis of 18 naturally occurring amino acids evaluated in this study was first assessed in positive ion mode and then in negative ion mode. Figure 2 shows a typical DESI MS spectrum for this analysis, in this case phenylalanine analyzed in (a) positive ion mode and (b) negative ion mode. Analysis in either mode reveals peaks corresponding to phenylalanine monomer and dimer species, observed as protonated analyte species in positive ion mode and deprotonated species in negative ion mode. Sodiated aa species were also observed in positive mode in some cases. Overall, for all the aas evaluated, the negative ion mode showed superior performance over the positive ion mode resulting in cleaner spectra with fewer chemical background peaks; possibly due to the inability of these background species to ionize in negative ion mode. Table 1 shows a comparison of the detection limits obtained for each amino acid in both positive and negative mode. For all the aas evaluated, the negative ion mode resulted in better detection limits than the positive ion mode except for the aas with basic side chain such as lysine and tryptophan, which show better performance in the positive mode due to

their high proton affinities. Aspartic and glutamic acid were only observed in the negative ion mode at the concentrations evaluated. The acidic nature of the molecules may be the cause of this phenomenon, as it would greatly impede the protonation of the molecules in positive mode. Tyrosine was not detected at these levels in either mode.

In an effort to increase the selectivity and hopefully the sensitivity in the detection of amino acids, and for potential applications of their detection in complex mixtures, the experiments were reevaluated in the reactive mode. Various cyclodextrins including α- and β-cyclodextrin were evaluated as chemical agents for the selective recognition of amino acids by complexation reactions. Only α-cyclodextrin was observed to form stable gas phase complexes with the amino acids which were detected in negative ion mode as the deprotonated complexes. Various concentrations of α-cyclodextrin were evaluated for the analysis of methionine standards, resulting in 30 μM α-cyclodextrin as the optimum concentration. Figure 3 shows representative reactive DESI MS spectra of (a) phenylalanine, (b) aspartic acid and (c) glutamic acid with the optimized conditions. Only one analyte peak is observed in all three examples, assigned as their corresponding complexes with α-cyclodextrin, thereby affording a very selective method for their detection. These assignments were verified by tandem MS analysis of the peaks corresponding to each of the amino acid:α-cyclodextrin complexes. All 18 amino acids investigated in this study were detected within the evaluated concentration levels using the reactive DESI MS approach. This also resulted to an overall increase in sensitivity for all the aas as shown by the lower detection limits, Table 1, except for the aas with basic side chains (lysine, histidine and arginine) which showed the best sensitivity in the reagentless analysis in positive ion mode.

Tandem MS analysis of the deprotonated amino acid:α-cyclodextrin complexes resulted in the liberation of the neutral aa with the deprotonated α-cyclodextrin as the major product ion in the spectrum. This suggests that the complexes are most likely formed in solution/droplets by interactions of the carbonyl group of the aas with the deprotonated α-cyclodextrin, through hydrogen bonding interactions. Further support for this hypothesis was verified by the observation of similar complexes with formic and aspartic acid following ESI experiments of α-cyclodextrin solutions containing these acids (Figure S-1) in full scan and MS/MS modes. The observation of peaks corresponding to these acids in the full scan reactive DESI spectra indicated the presence of these acids as impurities in the spray solvent. The presence of these carboxylic acid impurities in the spray solvent, competing for reaction with the α-cyclodextrin limits the amount of this reagent available for reaction with the analyte, potentially resulted in ionization suppression. As such all reactive DESI spectra acquired in full scan mode were acquired in the 1050-1500 *m/z* range (above the *m/z* for the carboxylic acid, impurity complexes) with AGC, or auto-gain control, on in order to improve the sensitivity for detection of the amino acid (analyte) complexes.

The performance of the optimized reactive DESI MS approach was evaluated for the simultaneous determination of an equimolar mixture of aas (leucine, isoleucine, methionine, phenylalanine, tyrosine, and valine) commonly encountered during the clinical evaluation of aa disorders. Initial evaluation of the relative gas phase stability of each of these aa following their respective break down curves suggested that each of these aa-α-cyclodextrin complexes showed similar gas phase stabilities indicating the potential for their simultaneos detection from an equimolar mixtures (Figure 4).

Simultanoeus detection of each of the 6 aa in equimolar mixture was performed by reactive DESI coupled to a QiT mass spectrometer in the full scan (Figure S-2) and MRM modes following various programmed transitions which resulted in the neutral loss of each of the individual aas in the mixture. The sensitivity for the detection of each of these aas was then compared with similar analysis on a QqQ mass spectrometer in full scan (Figure 5a) and in the precursor ion scan (*m/z* 971, with the neutral loss of each aa, Figure 5b). The full scan spectra collected with both instruments resulted in the unambiguous simultanoeus detection of all 6 aas investigated with the analysis performed on the QqQ instrument showing better sensitivity (see Table 2). The selectivity for the detection of each of the aas in the mixture was improved by performing the analysis in the MRM mode for the QiT and precursor ion scan in the QqQ instruments. In this case the QiT instrument showed superior sensitivity probably because the AGC capabilities of the QiT which allowed for the ion trap to selectively accumulate ions of interest during MRM experiments. Applications of this protocol for the direct determination of aas in human serum samples are currently underway.

CONCLUSIONS

The presented investigations indicate that DESI MS can serve as a rapid method for the detection of amino acids at concentrations comparable to those found in human serum samples. The detection limits were in the general regimes of the expected amino acid concentrations in normal human serum. Reagentless DESI MS analysis enabled the detection of all 18 aa investigated, however with limited sensitivity and selectivity, which was achieved by performing the investigation in the reactive DESI MS mode. The

optimized reactive DESI protocol enabled the simultaneous determination of a 6 aa commonly encountered during aa related disorders with the sensitivity for the detection of each aa improved by performing the analysis in the MRM mode when analysis is formed on a QiT and the precursor ion scan for analysis on a QqQ instrument. Further experiments are currently underway to adapt this analytical protocol for the direct determination of amino complex mixtures such serum samples.

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Figure Captions:

Figure 1: Schematic of the DESI set-up showing the sprayer, sampling platform, and mass spectrometer inlet capillary.

Figure 2: DESI spectra of 5 µL, 1 mM phenylalanine standards spotted onto PTFE coated glass slides and air dried prior to analysis in (a) positive ion mode and (b) negative ion mode.

Figure 3: Reactive DESI spectra using 30 µM α-cyclodextrin solvent spray for the analysis of amino acid standards (5 µL, 1 mM) that were deposited onto PTFE coated glass slides and air dried prior to analysis: (a) phenylalanine; (b) aspartic acid; (c) glutamic acid. The inserts show the corresponding MS/MS spectrum of each of the amino acid complexes with α-cyclodextrin.

Figure 4: Breakdown curves of various amino acid:α-cyclodextrin complexes including: valine, phenylalanine, methionine, leucine and isoleucine. 5 µL, 1 mM of each amino acid samples was spotted onto PTFE coated glass slides, air dried, then analyzed with a spray solution containing 30 µM of α-cyclodextrin in 50:50 MeOH:ACN.

Figure 5: Spectra obtained by spotting 5 µL of an equimolar (1 mM) mixture of amino acids including: valine, phenylalanine, methionine tyrosine, leucine and isoleucine onto PTFE coated glass slides, air dried, then analyzed with a spray solution of 30 µM of α-cyclodextrin in 50:50 MeOH:ACN. (a) full scan mode and (b) precursor ion scan of *m/z* 971.2. Experiments performed on the triple quadrupole mass spectrometer.

Table Captions:

Table 1: Limit of detection values based on individual amino acid standards. These are compared to literature values that are consistent with human serum levels. (*Calculated at signal-to-noise ≥ 3)

Table 2: Limit of detection values based on an equimolar (1 mM) mixture of amino acids including: valine, phenylalanine, tyrosine, leucine and isoleucine. The MRM limit of detection was found through a serial dilution of the amino acid mixture. These are compared to literature values that are consistent with human serum levels. (*Calculated at signal-to-noise ≥ 3). All experiments were run in negative mode.

Figures

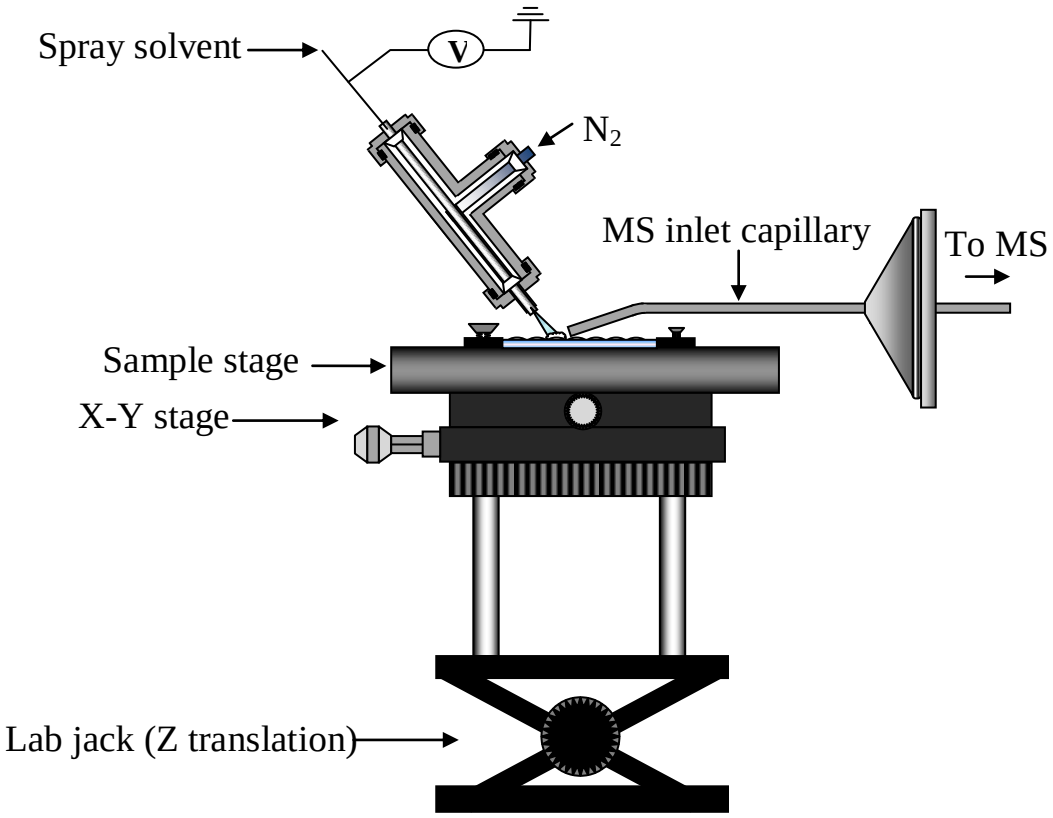

Figure 1

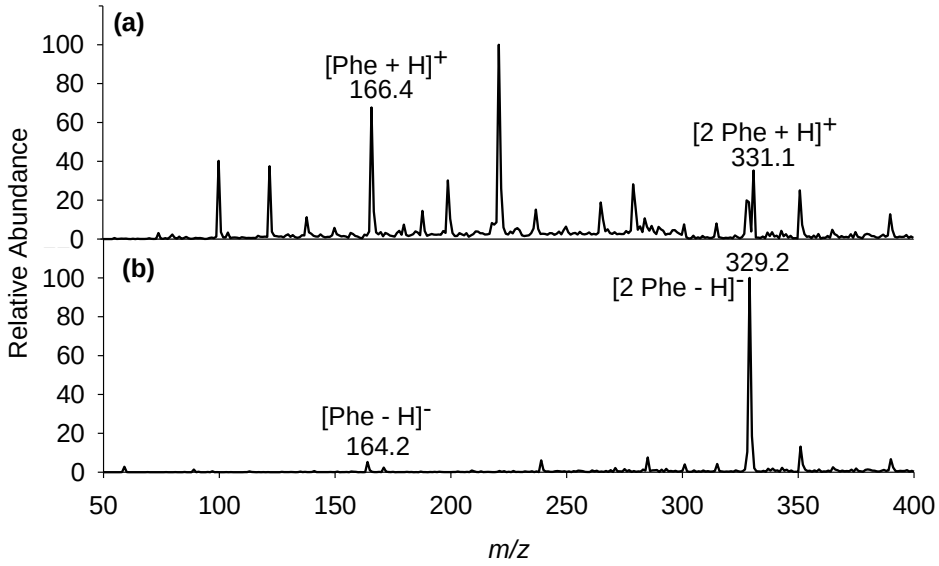
(a)
[Phe + H]+
166.4
[2 Phe + H]+
331.1
(b)
329.2
[2 Phe - H]-
[Phe - H]-
164.2
Relative Abundance
m/z

Figure 2

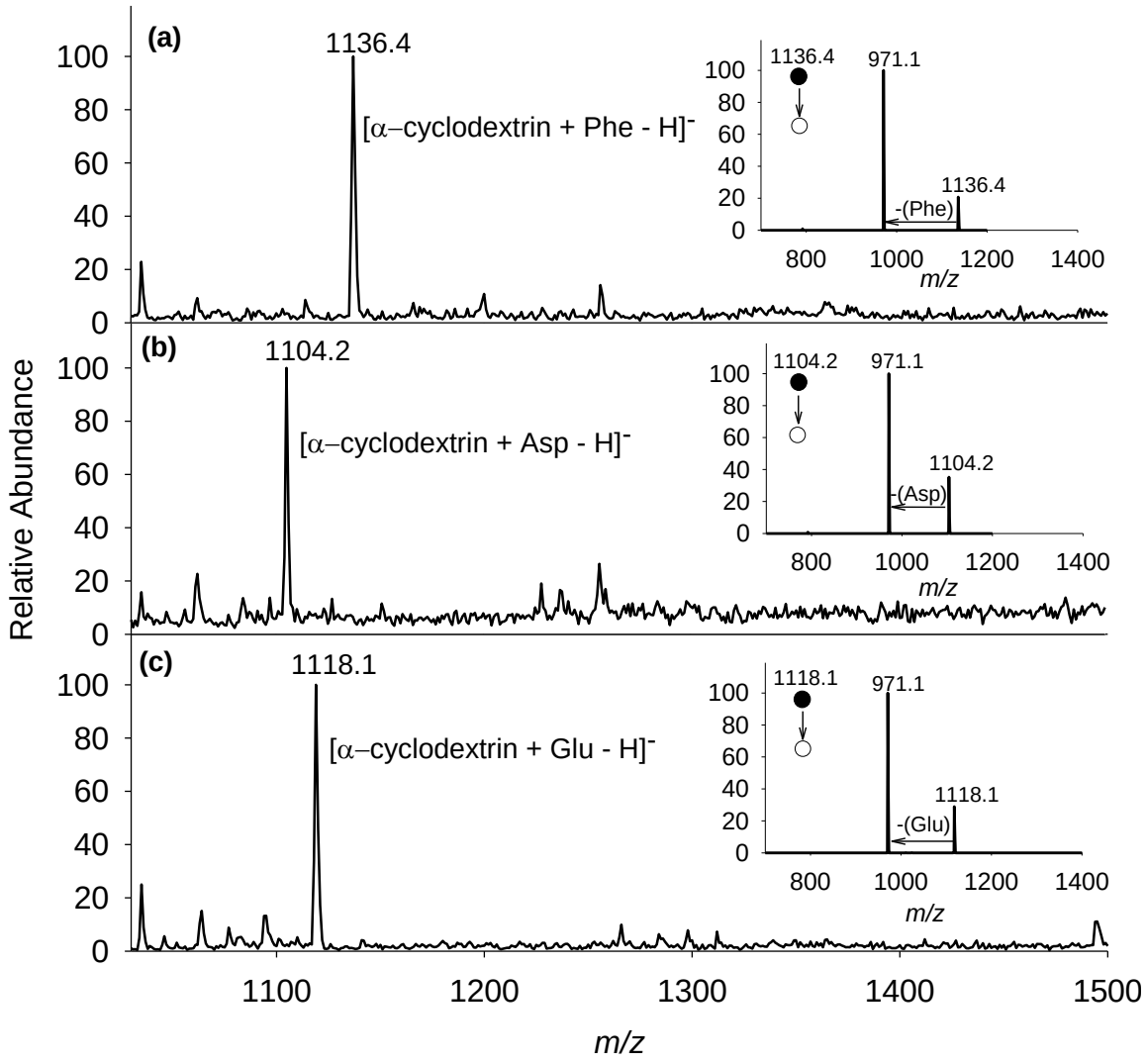
(a)
1136.4
[α–cyclodextrin + Phe - H]-
1136.4
971.1
1136.4
-(Phe)
(b)
1104.2
[α–cyclodextrin + Asp - H]-
1104.2
971.1
1104.2
-(Asp)
(c)
1118.1
[α–cyclodextrin + Glu - H]-
1118.1
971.1
1118.1
-(Glu)
Relative Abundance
m/z
m/z

Figure 3

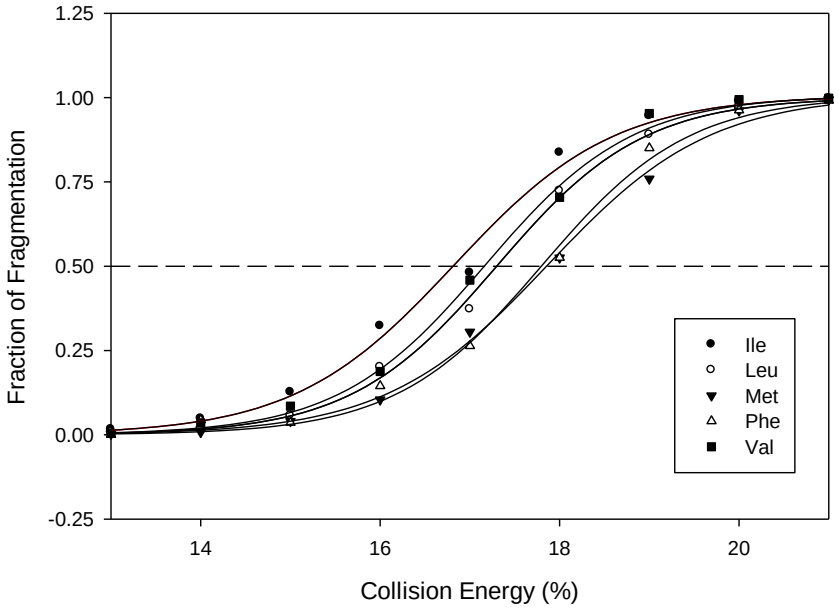
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
Fraction of Fragmentation
14
16
18
20
Collision Energy (%)
Ile
Leu
Met
Phe
Val

Figure 4

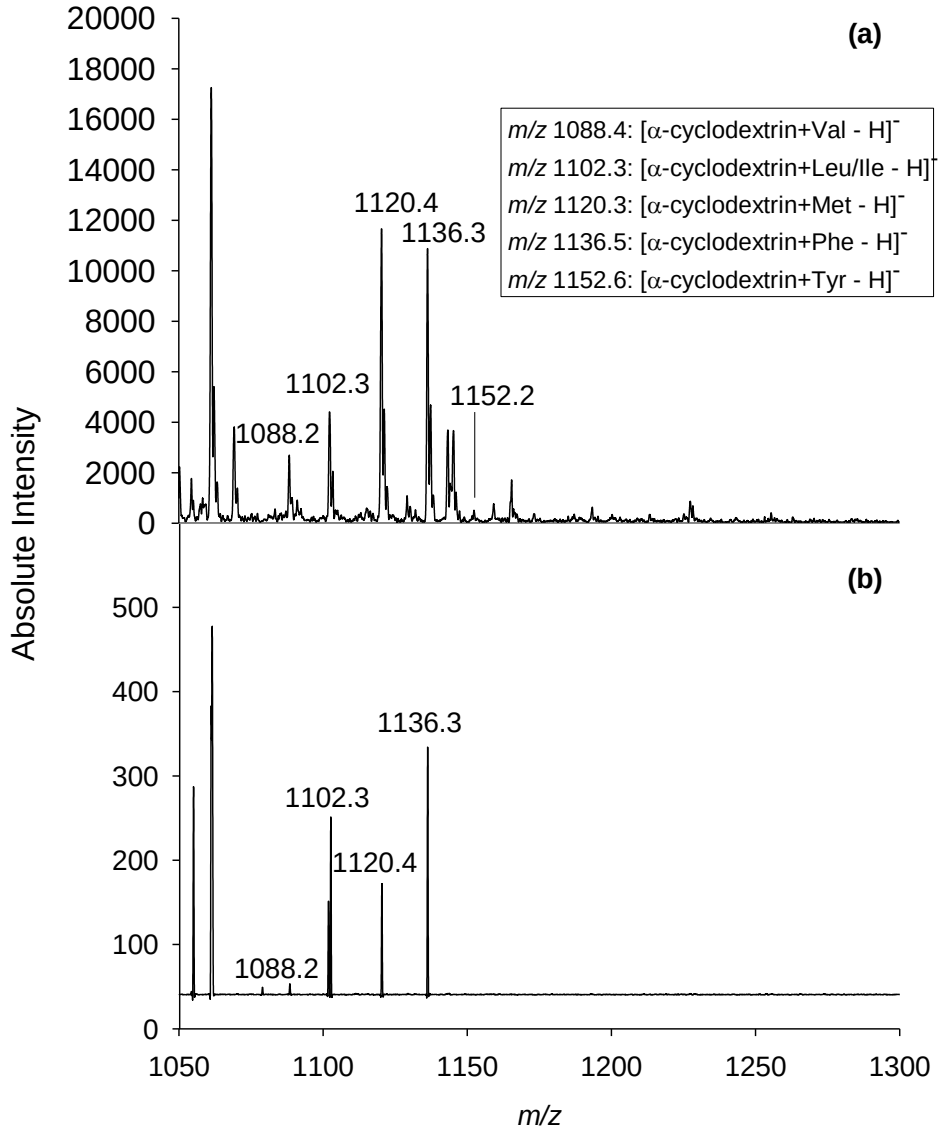
(a)
(b)
Absolute Intensity
m/z
m/z 1088.4: [α-cyclodextrin+Val - H]-
m/z 1102.3: [α-cyclodextrin+Leu/Ile - H]-
m/z 1120.3: [α-cyclodextrin+Met - H]-
m/z 1136.5: [α-cyclodextrin+Phe - H]-
m/z 1152.6: [α-cyclodextrin+Tyr - H]-
1088.2
1102.3
1120.4
1136.3
1152.2

Figure 5

Tables:

Table 1:

	QiT LOD (nmol)				
Amino Acid	**Pos. Mode Reagentless**	**Neg. Mode Reagentless**	**Neg. Mode Reactive**	**SRM Mode Reactive**	**Amount aa/ 5µL human serum (nmol)[18]**
Alanine	1.85	0.90	0.58	-	1.665 ± 0.370
Arginine	0.82	0.74	2.76	-	0.400 ± 0.100
Aspartic Acid	ND	0.83	0.52	-	0.015 ± 0.005
Glutamic Acid	ND	2.43	0.18	-	0.120 ± 0.075
Glutamine	7.05	4.14	0.91	-	2.930 ± 0.420
Glycine	2.65	2.24	0.44	-	1.150 ± 0.260
Histidine	0.35	0.30	4.15	-	0.410 ± 0.050
Lysine	0.52	1.04	0.91	-	0.940 ± 0.160
Proline	2.33	1.09	0.99	-	0.840 ± 0.300
Serine	3.96	0.58	0.50	-	0.570 ± 0.095
Threonine	7.13	0.90	0.55	-	0.700 ± 0.165
Tryptophan	2.06	3.25	0.77	-	0.220 ± 0.035
Isoleucine	3.51	2.07	0.26	0.19	0.310 ± 0.070
Leucine	3.96	2.52	0.35	1.95	0.615 ± 0.125
Methionine	1.98	0.62	0.85	0.31	0.125 ± 0.020
Phenylalanine	3.29	0.45	0.45	0.11	0.285 ± 0.045
Tyrosine	ND	ND	6.29	1.37	0.295 ± 0.060

Table 2:

	QiT LOD (nmol)		QqQ LOD (nmol)	
Amino Acid	**Full Scan**	**MRM**	**Full Scan**	**Precursor Ion Scan**
Isoleucine	2.15	0.134	0.36	1.21
Leucine				
Methionine	0.67	0.068	0.17	1.79
Phenylalanine	0.87	0.068	0.11	0.91
Tyrosine	2.89	0.002	2.20	ND
Valine	5.62	0.014	0.73	7.43

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